# The 'K' selected oligophilic bacteria: A key to uncultured diversity?

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**Molecular techniques have made it increasingly clear that a large proportion of bacterial diversity in natural habitats is uncultured and therefore unexplored. We suggest and give evidence in support of a hypothesis that a large proportion, if not all, of the uncultured diversity from a variety of aquatic and terrestrial habitats are oligophilic (oligotrophic) bacteria. Oligophilic bacteria grow only on dilute nutrient media and form small or microscopic colonies. A technique to cultivate and isolate the moderately oligophilic bacteria was developed and 90 cultures isolated. The twelve bacterial cultures characterized showed high growth yield coefficients and carbon conversion efficiencies at low substrate concentrations and progressively decreased with increasing substrate concentrations. Most of the growth yields were substantially higher than those reported in the literature and lie near the theoretical maximum. Slow growth rates and high yields indicate that they are 'K' selected species. 16S rDNA partial sequence analysis of the isolates indicates that it is a novel as well as diverse group.**

MUCH of the knowledge of classical microbiology has been contributed by pure culture techniques. It is becoming increasingly clear, however, that this stronghold of microbiology itself is its limitation, since a much greater fraction of the microbial diversity has never been cultivated and hence remains unexplored. It is known for a long time that there exists a difference of one to two orders of magnitude between direct microscopic count and colony count on any medium. This is known as the 'great plate count anomaly'[1–3]. In the last decade a number of molecular techniques have been employed to assess the bacterial diversity independent of culture methods[1,4–18]. These studies reveal that 99% or more of the bacterial diversity remains uncultured and therefore unexplored[4,14,19].

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The 'unculturability' is unlikely to be absolute. If they grow in nature they must grow under some set of laboratory conditions too. The unculturability must be our failure to identify conditions in which they grow. Throughout the last decade the number of published studies on bacterial diversity using culture independent molecular methods far outnumbers the ones attempting to culture the so far uncultured bacteria. Use of molecular methods is mostly limited to detection and identification of novel organisms. They do not give any phenotypic and functional information. Although it is possible in principle to clone the genes of an organism without culturing it[6], in the absence of phenotypic information obtaining novel enzymes, antibiotics and other useful biomolecules from these organisms is unlikely to be practicable and cost efficient. A cultural approach, therefore, will always prove better if successful.

Unculturability is also a phenomenon shown by well-studied species such as *Escherichia coli*, which enter a viable-non-culturable (VNC) state under certain conditions and the physiological reasons for this phenomenon are being investigated[20]. A sudden transfer to nutrient-rich media is believed to induce a metabolic imbalance resulting in death of a substantial proportion of cells[20]. What applies to VNC state of known organisms might also apply to the so-called unculturable species. It was realized many decades ago that the nutrient concentrations in the commonly used media are several fold higher than those present in their natural environments[3,21,22]. A number of bacterial isolates were shown to grow in extremely dilute culture media and failed to grow on conventional media[23–28]. These bacteria were called 'oligotrophic' bacteria. The terms oligotrophic and eutrophic are more popularly used to describe aquatic ecosystems. We would therefore prefer the word 'oligophilic' to describe organisms that grow only in nutrient-poor conditions in primary culture. A number of workers have shown that for oligotrophic environments plate counts on nutrient-poor media are several fold higher than those obtained on conventional media[23–26]. Further, a number of cultures were shown to grow on poor media but failed to grow on rich media at least on primary culture. However, many of the cultures could grow on conventional media after a few subcultures and therefore the definition of 'oligotrophic' bacteria was confused[25]. Many of the cultures were characterized morphologically and biochemically and a substantial proportion of isolates could not be identified with any of the existing phena[24,25]. Even the ones tentatively ascribed to some genera had important differences[24]. The oligophilic bacteria thus seemed to be composed of a large number of novel and diverse species.

In spite of the concept of oligophily being old, it is surprising that it has attracted little attention in the 1990s when bacterial diversity has become an active focus of research. With the possible exception of marine ultramicrobacteria[27] there has been little progress in studying microbial diversity using a cultural approach. We present evidence suggesting that oligophilic bacteria are abundant in a variety of aquatic and terrestrial environments and may constitute the majority of 'uncultured diversity'. A substantial proportion of them are culturable and can be isolated in pure culture using a simple protocol. The culturing protocol is based on the concept of providing a wide variety of substrates, but each one in an exceedingly small concentration. We show further that these bacteria are 'K' selected species employing different survival strategies than the fast growing 'r' selected bacteria.

A variety of soil, water and plant surfaces were sampled for the study. Out of the 16 water samples, 2 were from river Mutha which has substantial levels of pollution, 6 from garden reservoirs, 4 from nutrient-poor monsoon hill streams and 4 from moderately eutrophic lakes. Regarding the soil samples, 2 were from recreational gardens, 3 from agricultural fields, 2 from compost heaps, 2 from barren lands and 3 from forest soils. Other samples consisted of 2 leaf surfaces, 3 rock surfaces and 3 bark surfaces of living trees. All the samples were collected in sterile containers and plated out in less than 6 h of collection.

Two media were used in two different concentrations to plate out appropriate dilutions of the sample. (i) Standard Plate Count (SPC) medium (tryptone, 5 g; yeast extract, 2.5 g; NaCl, 2.5 g; glucose 10 g; distilled water 1 l; pH 7.0; agar agar, 20 g. (ii) Ravan medium (glucose, 5 g; peptone, 5 g; yeast extract, 5 g; sodium acetate, 5 g; sodium citrate, 5 g; pyruvic acid, 2 g; distilled water, 1 l; pH 7–7.2; agarose, 10 g). Both the media were used in the conventional or rich form and a 1:100 diluted or poor form.

All the soil samples were diluted serially to $1:10^6$ and all water samples to $10^3$ with distilled water and 0.1 ml each was plated out with rich and poor Ravan medium. Four soil and three water samples were simultaneously plated with rich and poor SPC media. For leaf, bark and rock surfaces chipped pieces of these surfaces with approximately 10 sq cm exposed area were shaken vigorously in 100 ml sterile distilled water in polypropylene flasks and 0.1 ml of the contents plated with rich and poor Ravan media. All the plating was in triplicates. The plates were incubated in moist chambers at ambient temperatures for 21 to 36 days. Colonies appearing on rich media were counted after 48 h but continued to be incubated for three more weeks and counted again. A 48 h count was necessary since often a spreading fungal or bacterial colony overgrew the plate obscuring other colonies on prolonged incubation. This problem was not encountered on poor media.

Counts in all the plates were taken visually as well as microscopically. This was essential since a large pro-

portion of colonies on poor medium were microscopic (15 to 100 microns in diameter). Microcolonies in 25 low power fields were counted and extrapolated to the entire area of the plate. In order to avoid edge effect, only the colonies whose center was in the field were included.

Microcolonies growing on poor Ravan media were observed and picked up under a stereoscopic microscope with sterile glass capillaries and suspended in 0.1 ml sterile saline. Each of the suspensions was streaked on rich and poor Ravan media simultaneously. The plates were incubated at room temperatures for 2 to 14 days. After visible or microscopic colonies appeared, each colony was streaked on poor and rich Ravan media again till a reliable pure culture was obtained. Both rich and poor media were used at each subculture but growth only from poor medium was used for subculture. All cultures were tested for their ability to grow on a low concentration of glucose as a sole source of carbon and energy. Out of the 63 cultures that could utilize glucose, 11 colonies with apparent morphological differences were selected for growth curve and growth yield studies as well as 16 S rDNA sequencing. In addition, one fungal and one actinomycete isolate were subject to growth characterization and identified morphologically.

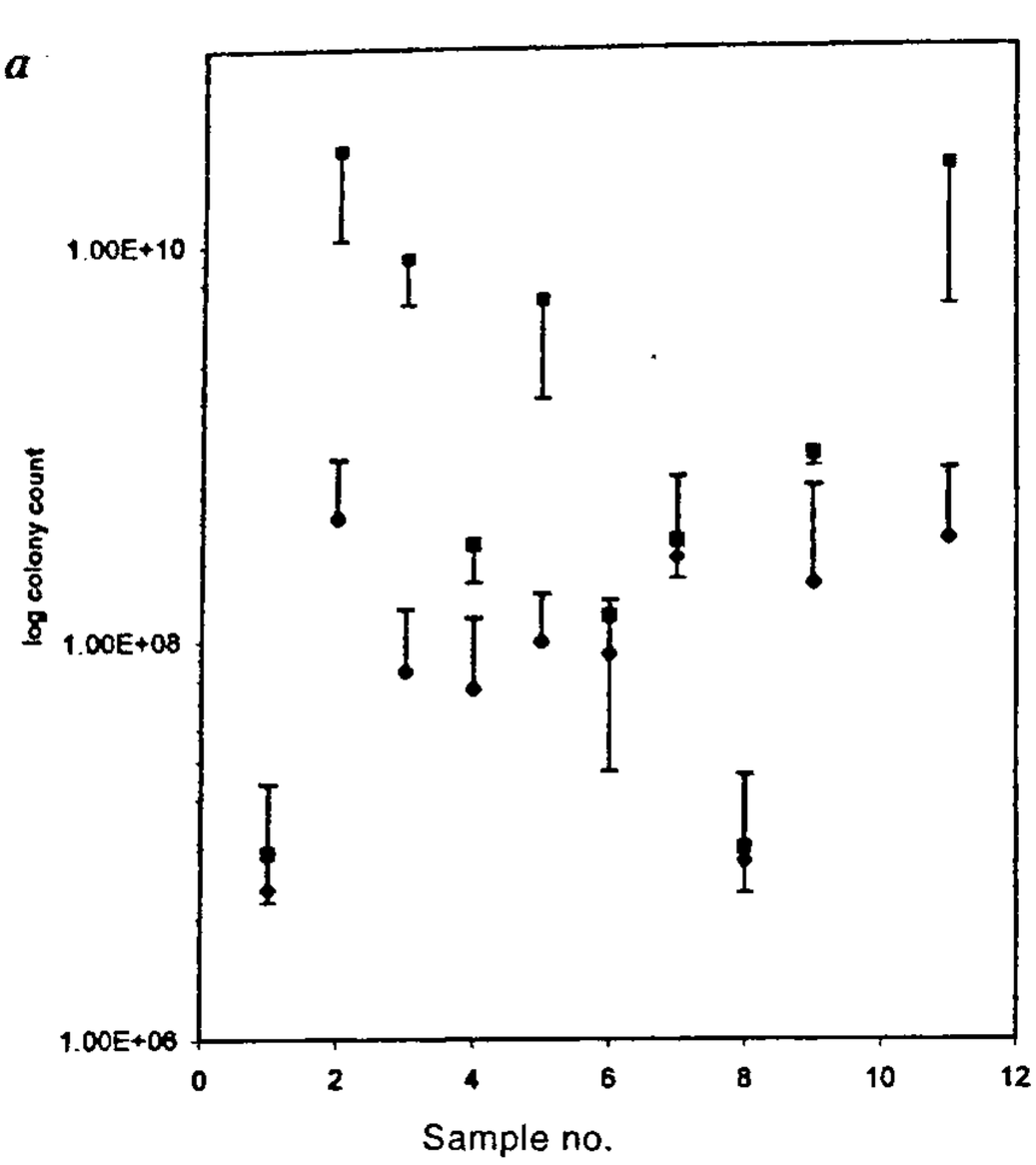
To compare growth at low and high sugar concentrations, three flasks containing 100 ml each of 0.05, 0.1 and 1.0 g% glucose in a basal salt medium (sodium nitrate, 2 g; dipotassium hydrogen phosphate, 1 g; potassium dihydrogen phosphate, 0.5 g; distilled water, 1 l) were inoculated with a single colony and incubated on a rotary shaker for six days. Absorbance at 530 nm was recorded after every 12 h.

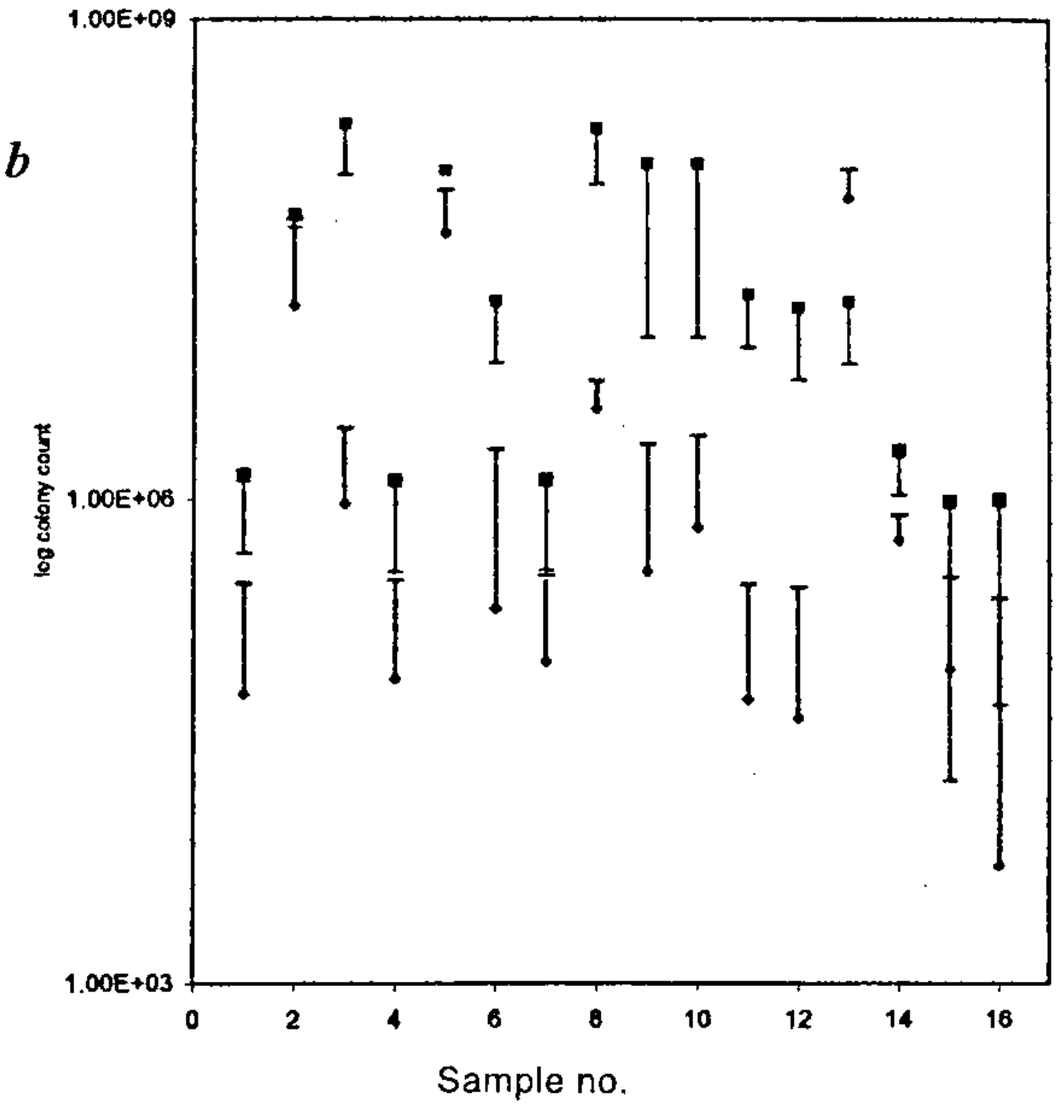
To compare the growth yield at low and high substrate concentrations, the above cultures were inoculated in minimal medium with glucose in concentrations of 1, 10, 100 and 1000 mg/dl along with a control without glucose. After sterilization of the medium the initial sugar concentration was determined using dinitrosalicylic acid[28]. The flasks were incubated at room temperature in the dark to eliminate photophosphorylation if any. After ten days the residual sugar concentration, the dry weight of the biomass and the total cell protein by the Folin Lowry method[28] were estimated. Along with the oligophilic cultures dry mass of *E. coli* grown in LB medium was used as a standard for cell protein estimation. The protein content estimated was converted into biomass by multiplying with the ratio of dry biomass to protein content of the *E. coli* standard. If a difference was observed between the dry weight obtained by gravimetry and that by protein estimation, the smaller of the two was taken for statistical analysis. For every culture the experiment was replicated thrice.

The genomic DNA was isolated from pure bacterial cultures by the standard chloroform/iso amyl alcohol procedure[29]. The amplification of 16S rRNA gene was carried out using PCR (ref. 29) in a DNA thermal cycler (MJ Research, PTC 200). Two universal primers used in amplification had the sequence 5'-AGAGTTTGATCHTGGCTCAG-3' (F 27) and 5'-TTCTGCAGTCTAGAAGGAGGTG-(T/A)TCCAGCC (R1525 XP), and correspond to positions 8–27 and 1525–1554 respectively, in the 16S rDNA sequence of *E. coli*.

A 1.5 kb fragment of 16S rRNA gene was amplified and purified by PEG-NaCl precipitation. The purified product was sequenced directly by Gibco-BRL ds Cycle Sequencing kit using the manufacturer's protocol. The gel was run on IBI-Kodak Base Runner 100 and an electrolyte gradient was used to enable reading of longer sequences[29]. Using a single primer that binds to position 343–358 (*E. coli* numbering), the sequence of 350 bases at the 5′ end of the molecule was determined. The primer used was R-343 and had the sequence 5′-ACTGCTGCCTCCCGTA-3′. The sequence was read manually and analysis was done using the RDP (Ribosomal Database Project)[30]. The analysis gave a partial 16S rRNA sequence of various isolates. The alignment, similarity and distance estimations were done using CLUSTAL W (ref. 31). The similarity coefficient ($S_{ab}$) values were obtained by similarity check program at RDP.

Colony counts on rich and poor media were significantly different in 6 out of 11 soil samples, 12 out of 16 water samples and all the surface samples (Figure 1). Counts on rich Ravan and rich SPC media were not significantly different but for four out of seven samples microcolony counts on poor Ravan medium were up to three-fold greater than those on poor SPC media (data not shown). More than 90% of the colonies on poor media were microscopic ranging from 15 to 100 microns in diameter which appeared between the second and the twenty-fourth day and did not grow in size on prolonged incubation (Figure 2). The microcolonies on poor medium outnumbered the visible ones on poor medium as well as rich medium by up to two orders of magnitude. From soil and water plates a randomly chosen 200 microcolonies were picked up with glass capillaries and plated onto rich and poor Ravan media simultaneously. The ones failing to grow on rich medium but growing on poor media were selected for further subcultures. The 156 selected cultures were subject to six subsequent subcultures on rich and poor Ravan media. Out of 156 cultures, 66 failed to grow on both the media on second or third subculture. The remaining 90 cultures grew well on poor medium and started growing on rich medium between the second and fourth subculture. By the fourth subculture all of them could grow on both rich and poor media. The colony size on poor medium gradually increased and the incubation time needed for appearance of colonies decreased. By the fourth or fifth subculture all colonies were visible on the third or fourth day and ranged between 0.4 and 1.5 mm in size.



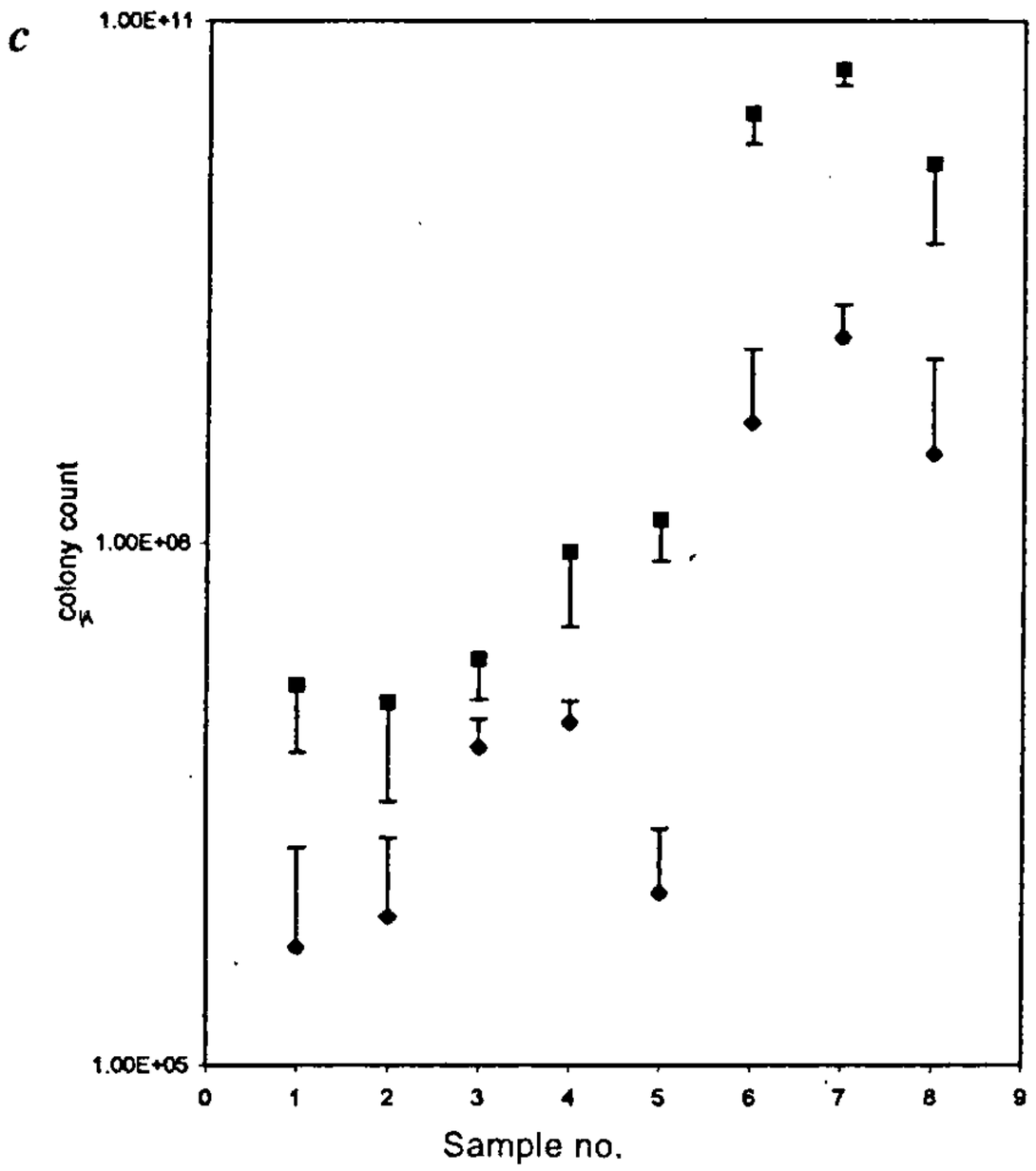




**Figure 1.** Plate counts on poor (squares with downward error bars) and rich (diamonds with upward error bars) media. The bars indicate confidence intervals based on 't' distribution. *a*, Soil samples coming from garden (2), agricultural fields (2), compost heaps (2), barren land (2) and forest (3) in sequence. *b*, Water samples coming from high BOD river (2), garden tanks and fountains (6), monsoon hill streams (4) and moderately eutrophic lakes (4) in sequence. *c*, Samples from rock surface (3), leaf surface (2) and bark (3) in sequence.

The 16S rDNA sequence analysis of the hypervariable region was done. This region has been used successfully for molecular taxonomy studies[32,33]. The results showed that none of the isolates matched with any of the sequences in the database to a species level (Table 1). The maximum similarity was shown by isolate M50 with

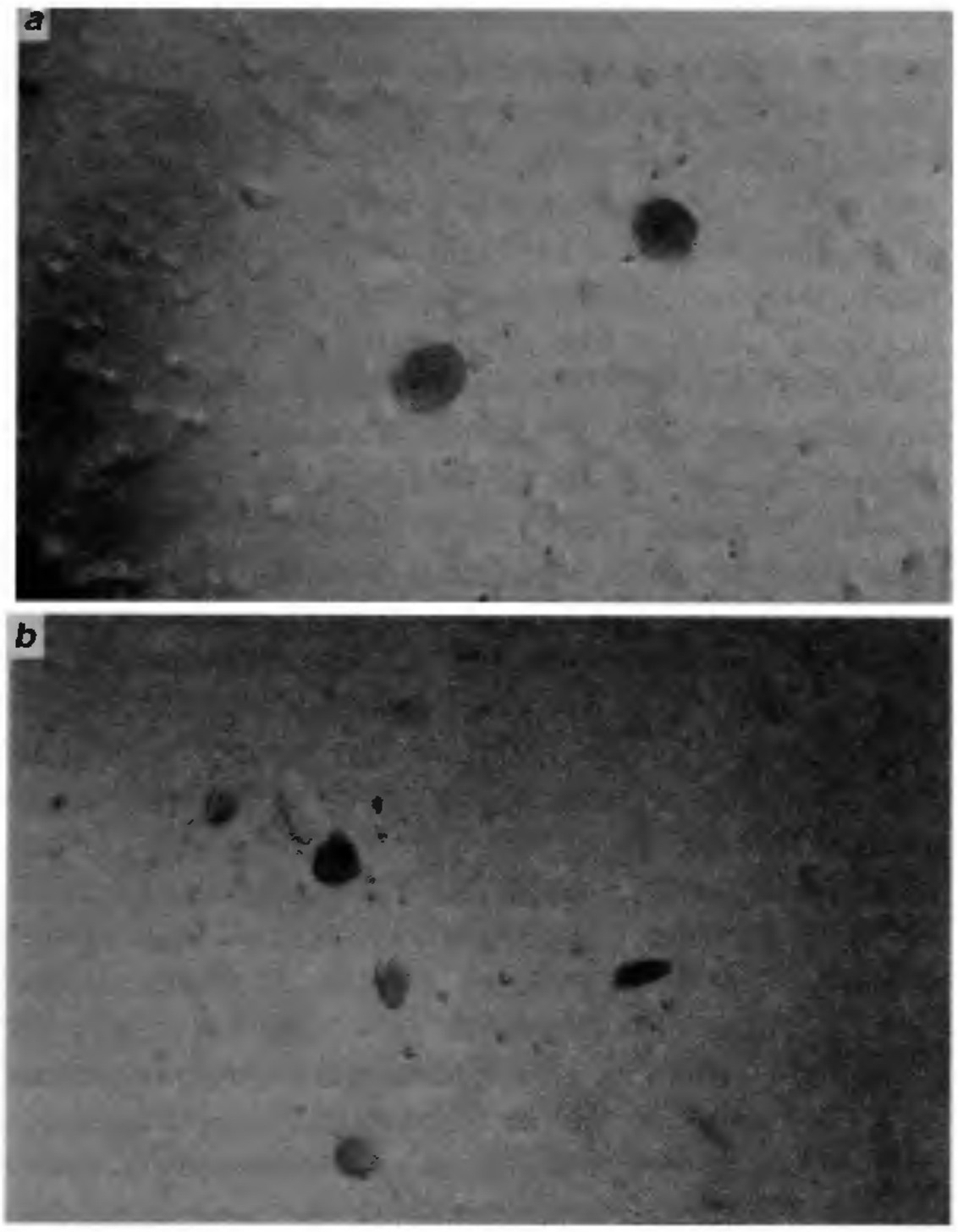



**Figure 2** *a*, *b*. Microcolonies on poor medium after 3 weeks incubation. The bar represents 100 micron.

**Table 1.** Maximum sequence similarities of the isolates with the RDP database

| Name of culture | Sequence matches found in RDP | $S_{ab}$ value |
|---|---|---|
| N1 | Clone JAP403 | 0.568 |
| N15 | *Acinetobacter* sp. str. UNIP2 | 0.602 |
| | *Acinetobacter calcoaceticus* DSM 30006 (T) | 0.602 |
| | *Acinetobacter calcoaceticus* DSM 1139 | 0.602 |
| N30 | *Halomonas marina* str. DSM 4741(T) | 0.449 |
| N37 | *Cytophaga uliginosa* NCIMB 1863 | 0.320 |
| N43 | *Pseudomonas putida* IFO 14614 | 0.534 |
| | *Pseudomonas putida* IAM 1236 | 0.534 |
| C1 | *Paenibacillus kobensis* IFO 15729 (T) | 0.654 |
| C2 | *Porphyrobacter neustonensis* ACM 2844 | 0.434 |
| C3 | *Pseudomonas chlororaphis* IAM 12354 | 0.700 |
| | *Azospirullum* sp. str. AM-53 DSM 1727 | 0.695 |
| M7 | *Bacillus licheniformis* NCDO 1772 | 0.621 |
| M33 | Str. Dros CA | 0.336 |
| | *Escherichia coli* 9 | 0.336 |
| M50 | *Acinetobacter* sp. str. UNIP2 | 0.735 |
| | *Acinetobacter calcoaceticus* DSM 30006(T) | 0.735 |
| | *Acinetobacter calcoaceticus* DSM 1139 | 0.735 |

*Acinetobacter* sp. (0.735). The isolate C3 resembled *Pseudomonas chlororaphis* by 0.7. However, unlike both the genera, isolates M50 and C3 were gram positive cocci. Other isolates had poor similarity values with the sequence data.

Although all the isolates ultimately adapted to conventional media, the growth yields at lower substrate concentrations were substantially greater than those at higher concentrations (Figure 3). The response to high substrate concentration was varied. The isolate N43, for example, showed aborted growth at 1 g% sugar whereas C3 had a prolonged lag. During the lag, glucose was being consumed but no growth was apparent. The rapid consumption of glucose decreased the concentration to 26 mg% after which the cell density started increasing. Most of the isolates showed arithmetic rather than exponential growth that is characteristic of bacteria in batch culture (data not shown).

Oligophiles are known to constitute the majority of marine bacterial communities[23,25,27]. Studies on oligophiles of soil and freshwater are scanty[26] but confirm their presence. The differences in counts on rich and poor media from a variety of aquatic and terrestrial habitats indicate that oligophilic bacteria are widely distributed in aquatic and terrestrial habitats too. Oligophiles were found to account for up to 90% of the direct microscopic counts[25] almost eliminating the 'great plate count anomaly'. Although we did not compare the plate counts with direct microscopic counts, the difference of one or two orders of magnitude in plate count suggests that 90 to 99% of bacteria in these habitats are perhaps oligophilic. The marine extreme oligophiles were reported to be inhibited by > 5 mg/l concentration of amino acids[27]. Our isolates as well as those of Whang and Hattori[26] were obtained at 200 to 270 mg of organic

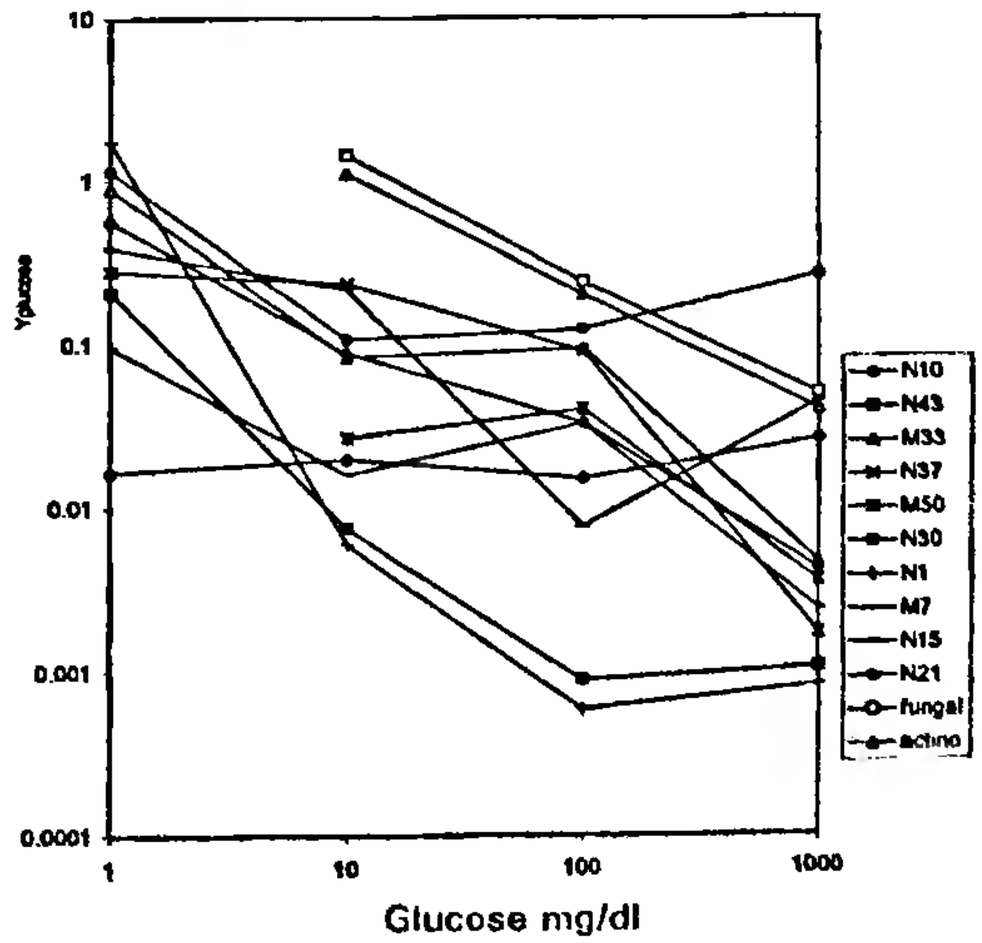



**Figure 3.** Growth yield (mg biomass/mg sugar consumed) at increasing sugar concentrations. Error bars omitted for simplicity.

substrates per litre and could be regarded as moderate oligophiles. It is noticeable that a significant difference in counts could be obtained in moderately polluted waters as well as root zone soils and compost heaps which are not considered to be typically oligotrophic environments. The oligophiles therefore do not seem to be restricted to extreme oligotrophic marine habitats. A large number of moderate oligophiles inhabit natural habitats with varying trophicity.

A source of confusion is that most of the moderate oligophiles get adapted spontaneously to conventional media after repeated subcultures and therefore for a given isolate oligophily is difficult to demonstrate reproducibly. Whether the adaptation is due to a physiological switch or mutation-selection remains to be studied. We found that although they adapt to conventional media, the growth yields at nutrient concentrations of conventional media remain relatively poor. In the classical model by Monod[33,34], the growth yields are independent of substrate concentration and a number of organisms are known to obey this rule over a wide range of substrate concentrations[34–38]. The oligophiles, on the other hand, showed a clear trend of reduction in growth yields with increasing substrate concentrations. This gives a clearer and reproducible working definition for oligophiles as organisms which do not grow or grow with reduced yields at concentrations of organic carbon above 1 g/l. It also indicates that the unculturability of oligophiles on conventional media on primary culture and the VNC state of many non-oligophiles are likely to be fundamentally different phenomena.

For 8 isolates the maximum growth yields obtained at 1 or 10 mg% of sugar were substantially higher than those reported for non-oligophilic heterotrophic bacteria so far. Since glucose served as the only source of carbon as well as energy, the organisms have to use part of it for energy generation and the rest for biosynthesis[39]. Maximum yield is possible when the division between these two channels is optimum. If a large amount of substrate is diverted to catabolism, biomass synthesis will be carbon limited and if a small amount is catabolized it will be energy limited. An optimum will be reached when the number of ATP generated are just sufficient to convert the entire residual substrate carbon into biomass, i.e.

$$CAY_{ATP} = (1-C) \times \text{Mol wt. of glucose} \times \text{carbon fraction in glucose/carbon fraction in biomass},$$

where $C$ is the fraction of glucose catabolized and $A$ is the moles of ATP per mole of glucose catabolized.

We assume a limiting case here where there is no assimilatory carbon dioxide release and the entire available carbon is converted into biomass.

For glucose as a substrate, and assuming that carbon constitutes 50% of the dry biomass,

$$CAY_{ATP} = 144 \times (1-C).$$

This condition is satisfied when

$$C = 144/(AY_{ATP} + 144).$$

At this optimum value of $C$, the maximum biomass production can be calculated as $225 \times (1-C)$. At $Y_{ATP}$ of 10 g/mole which is the average for a number of heterotrophic species[36,38,40], and assuming aerobic respiration, producing 30 ATP/glucose, a maximum of 710 mg biomass can be produced from 1 g of glucose. The reported biomass yields of a number of non-oligophilic heterotrophic organisms is much smaller than this[34–38,40–42]. The oligophiles produced quantities of dry biomass near or above the theoretical maximum (Figure 4). Even if we take the lower limits of the confidence intervals, the growth yields are surprisingly high. For non-oligophilic heterotrophs, $Y_{ATP}$ up to 28.5 has been reported[41]. Oligophiles appear to have much larger $Y_{ATP}$. We have not probed into the mechanisms behind the high efficiency of carbon conversion. Presumably the metabolism of oligophiles at small substrate concentrations is radically different[38].

The high efficiency at low concentrations has a clear ecological implication. The oligophilic bacteria are slow growers and therefore their survival strategies in competition with fast growing heterotrophs have to be dif-

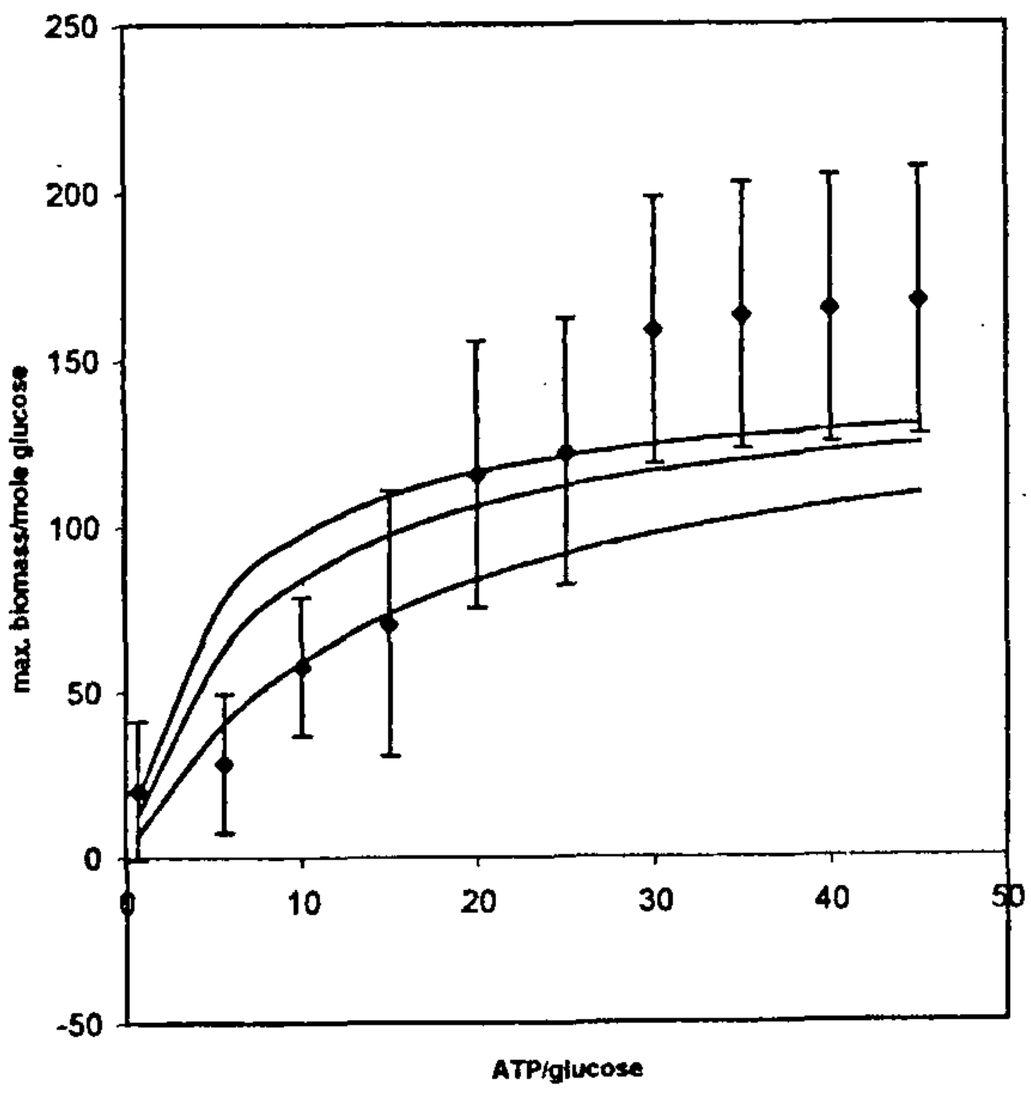



**Figure 4.** Maximum growth yield (determined at 10 mg/dl of sugar) of the isolates arranged in increasing order. The lines represent the theoretical maximum yield at increasing yields of ATP/mole of glucose. The three lines are computed for $Y_{ATP}$ of 10, 20 and 30 g/mole. The *E. coli* control did not give detectable growth at 10 mg/dl. At 100 mg/dl it yielded < 70 mg/mole sugar consumed.

ferent. Ecologists differentiate between the 'r' selected species which are opportunistic fast growers as against the 'K' selected species which may reproduce slowly but make a more efficient use of the environment and enjoy a larger carrying capacity[42–44]. The oligophiles fit into the role of the K selected species that enjoy better fitness at saturated or marginally life supporting conditions utilizing the minimum available resources more efficiently.

The sequence analysis, although preliminary at this stage, indicates that the group of isolates is novel as well as diverse. Although a small number of isolates were characterized, the small sample gives sufficiently strong indication that a substantial proportion of bacterial diversity can be cultured. Perhaps the most important implication of the study is that the cultural approach of classical microbiology is not obsolete in studying the so far unexplored diversity of micro-organisms. The novelty and diversity of the isolates coupled with the colony count differences indicate that a large proportion of the uncultured diversity might be made up of oligophiles. This also means that a substantial proportion of the so-called unculturable diversity can in fact be cultured with a simple and unsophisticated protocol. Research on oligophiles was active from the 1940s to 1970s giving ample indications that a large number of species await discovery. At that time molecular tools for taxonomic and phylogenetic analysis were not available and with phenotypic characterization a number of isolates were ascribed to existing genera despite some differences[24,25]. We suspect that some of the isolates, which superficially resembled these genera may in fact belong to different lineages. In addition, a large number remained unidentified. For some unknown reasons, research on oligophile diversity waned and not many papers are to be found in the 1980s and 1990s. We feel the need to revive this line of work in pursuit of the unexplored diversity not only in the marine and freshwater oligotrophic environments, but in a variety of other aquatic and terrestrial ones. A good combination of cultural and molecular approaches would certainly prove superior as well as cost effective compared to the culture independent molecular approach to explore microbial diversity.


1. Amann, R. I., Ludwig, W. and Schleifer, K., *Microbiol. Rev.*, 1995, **59**, 143–169.
2. Staley, J. T. and Konopka, A., *Annu. Rev. Microbiol.*, 1985, **39**, 321–346.
3. Roszak, D. B. and Colwell, R. R., *Microbiol. Rev.*, 1987, **51**, 365–379.
4. Torsvik, V., Goksoyr, J. and Daae, F. L., *Appl. Environ. Microbiol.*, 1990, **56**, 782–787.
5. Ward, D. M., Weller, R. and Bateson, M. M., *Nature*, 1990, **345**, 63–65.
6. Seow, K. T., Meurer, G., Wendt-Pienkowski, E., Hutchinson, C. R. and Davies, J., *J. Bacteriol.*, 1997, **179**, 7360–7368.
7. Polz, M. F. and Cavanaugh, C. M., *Appl. Environ. Microbiol.*, 1997, **63**, 1028–1033.
8. Field, K. G., Gordon, D., Wright, T., Rappe, M., Urback, E., Vergin, K. and Giovannoni, S. J., *Appl. Environ. Microbiol.*, 1997, **63**, 63–70.
9. Woese, C. R., *Curr. Biol.*, 1996, **6**, 1060–1063.
10. Weidner, S., Arnold, W. and Puhler, A., *Appl. Environ. Microbiol.*, 1996, **62**, 766–771.
11. Ohkuma, M. and Kudo, T., *Appl. Environ. Microbiol.*, 1996, **62**, 461–468.
12. Rheims, H., Sproer, C., Rainey, F. A. and Stackebrandt, E., *Microbiology*, 1996, **142**, 2863–2870.
13. Felske, A., Akkermans, A. D. and DeVos, W. M., *Appl. Environ. Microbiol.*, 1998, **64**, 4581–4587.
14. Bintrim, S. B., Donohue, T. J., Handelsman, J., Roberts, G. P. and Goodman, R. M., *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 277–282.
15. Bowman, J. P., McCammon, S. A., Brown, M. V., Nichols, D. S. and McMeekin, T. A., *Appl. Environ. Microbiol.*, 1997, **63**, 3068–3078.
16. Torsvik, V., Daae, F. L., Sandaa, R. A. and Ovreas, L., *J. Biotechnol.*, 1998, **64**, 53–62.
17. Liu, W. T., Marsh, T. L., Cheng, H. and Forney, L. J., *Appl. Environ. Microbiol.*, 1997, **63**, 4516–4522.
18. Hugenholtz, P., Pitulle, C., Hershberger, K. L. and Pau, N. R., *J. Bacteriol.*, 1998, **188**, 366–376.
19. Fuhrman, J. A. and Campbell, L., *Nature*, 1998, **393**, 410–411.
20. Bloomfield, S. F., Stewart Gordon, S. A. B., Dodd C. E. R., Booth, I. R. and Power, G. M., *Microbiology*, 1998, **144**, 1–2.
21. Zobell, C. E. and Grant, C. W., *Science*, 1942, **96**, 189.
22. Zobell, C. E. and Grant, C. W., *J. Bacteriol.*, 1943, **45**, 555–564.
23. Akagi, Y., Taga, N. and Simidu, U., *Can. J. Microbiol.*, 1977, **23**, 981–987.
24. Mallory, L. M., Austin, B. and Colwell, R. R., *Can. J. Microbiol.*, 1977, **23**, 733–750.
25. Kuznetsov, S. I., Dubinina, G. A. and Lapteva, N. A., *Annu. Rev. Microbiol.*, 1979, **33**, 377–387.
26. Whang, K. and Hattori, T., *Antonie van Leeuwenhoek*, 1988, **54**, 19–36.
27. Button, D. K., Frits, S., Pham, Q., Ravonna, M. and Betsy, R. R., *Appl. Environ. Microbiol.*, 1993, **59**, 881–891.
28. Plummer, T. D., in *An Introduction to Practical Biochemistry*, Tata McGraw Hill, 1988.
29. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G, Smith, J. A. and Struhl, K., in *Current Protocols in Molecular Biology*, John Wiley, New York, 1998.
30. Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J. and Woese, C. R., *Nucleic Acids Res*, 1996, **24**, 82–85.
31. Higgins, D. G., Bleasby, A. J. and Fuchs, R., *Appl. Biosci.*, 1992, **8**, 189–191.
32. Moore, E. R. B., Wittich, R. M., Fortnagel, P. and Timmis, K. N., *Lett. Appl. Microbiol.*, 1993, **17**, 115–118.
33. Monod, J., *Ann. Inst. Pasteur.*, 1942, **68**, 444.
34. Monod, J., *Ann. Rev. Microbiol.*, 1949, **3**, 371–394.
35. Bauchop, T. and Elsden, S. R., *J. Gen. Microbiol.*, 1960, **23**, 457–469.
36. Mayberry, W. R., Prochazka, G. J. and Payne, W., J., *Appl. Microbiol.*, 1967, **15**, 1332–1338.
37. Stouthamer, A. H., in *Methods in Microbiology* (eds Norris, J. R. and Ribbons, D. W.), Academic Press, London, 1969, vol. 1, pp. 629–664.
38. Gommers, P. J. F., van Schie, B. J., van Dijken, J. P. and Kuenen, J. G., *Biotechnol. Bioeng.*, 1988, **32**, 86–94.

39. Mainzer, S. E. and Hempfling, W. P., *J. Bacteriol.*, 1976, **126**, 251–256.
40. Stouthamer, A. H. and Bettenhaussen, C. W., *Arch. Microbiol.*, 1976, **111**, 21–23.
41. de Vries, W., Rietveld-Struijk, R. M. and Stouthamer, A. H., *Antonie van Leeuwenhoek*, 1977, **43**, 153–167.
42. MacArther, R. H. and Wilson, E. O., in *The Theory of Island Biogeography*, Princeton University Press, New Jersey, 1967.
43. King, C. E. and Anderson, W. W., *Am. Nat.*, 1971, **105**, 137–156.
44. Emlen, J. M., in *Population Biology: The Convolution of Population Dynamics and Behaviour*, MacMillan, New York, 1984.

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